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Occurrence and persistence of *Bacillus thuringiensis* (Bt) and transgenic Bt corn *cry1Ab* gene from an aquatic environment

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Abstract

Genetically modified corn crops and suspensions of *Bacillus thuringiensis* (Bt) are currently used to control pest infestations of insects of the Lepidoptera family. For this purpose, the *cry1Ab* gene coding for protein δ-endotoxin derived from *B. thuringiensis kurstaki* (Btk), which is highly toxic to these insects, was inserted and expressed in corn. The aims of this study were to examine the occurrence and persistence of the *cry1Ab* gene from Btk and Bt corn in aquatic environments near fields where Bt corn was cultivated. First, an optimal DNA preparation and extraction methodology was developed to allow for quantitative gene analysis by real-time polymerase chain reaction (qPCR) in various environmental matrices. Second, surface water and sediment were spiked in vitro with genomic DNA from Bt or Bt corn to evaluate the persistence of *cry1Ab* genes. Third, soil, sediment, and water samples were collected before seeding, 2 weeks after pollen release, and after corn harvesting and mechanical root remixing in soils to assess *cry1Ab* gene content. DNA was extracted with sufficient purity (i.e., low absorbance at 230 nm and absence of PCR-inhibiting substances) from soil, sediment, and surface water. The *cry1Ab* gene persisted for more than 21 and 40 days in surface water and sediment, respectively. The removal of bacteria by filtration of surface water samples did not significantly increase the half-life of the transgene, but the levels were fivefold more abundant than those in unfiltered water at the end of the exposure period. In sediments, the *cry1Ab* gene from Bt corn was still detected after 40 days in clay- and sand-rich sediments. Field surveys revealed that the *cry1Ab* gene from transgenic corn and from naturally occurring Bt was more abundant in the sediment than in the surface water. The *cry1Ab* transgene was detected as far away as the Richelieu and St. Lawrence rivers (82 km downstream from the corn cultivation plot), suggesting that there were multiple sources of this gene and/or that it undergoes transport by the water column. Sediment-associated *cry1Ab* gene from Bt corn tended to decrease with distance from the Bt cornfield. Sediment concentrations of the *cry1Ab* gene were significantly correlated with those of the *cry1Ab* gene in surface water ($R = 0.83$; $P = 0.04$). The data indicate that DNA from Bt corn and Bt were persistent in aquatic environments and were detected in rivers draining farming areas.

Keywords: Gene detection; Transgenic bt corn; Real-time polymerase chain reaction; Aquatic ecosystems; *cry1Ab* gene

1. Introduction

The insecticidal properties of δ-endotoxin (encoded in *cry1Ab* gene) from the bacterium *Bacillus thuringiensis* (Bt) have been recognized commercially for over 40 years (Porcar and Juarez-Pérez, 2003). As a Gram-positive aerobic bacterium, Bt has the ability to synthesize a crystalline protein (so called δ-endotoxins) during the sporulation process. The δ-endotoxin is a protein that is toxic to insects of the Coleoptera, Diptera, and Lepidoptera families. After ingestion by insects, the alkaline environment of the gut and proteases dissolve the crystalline proteins to yield the toxic form which leads to pore formation in the intestinal wall leading to severe inflammation, starvation, and death. In the mid-1990s, transgenic corn was genetically modified to express the toxic form of Cry1Ab toxin from *B. thuringiensis kurstaki* (Btk) in all its components. The transgene was designed for sustained expression by including the 35S promoter from a cytomegalovirus (CMV) and flanked by intron 1 of the corn-specific heat-shock protein 70 at the 5′ site (Zimmermann

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et al., 1998). Hence, genetically modified corn crops were marketed for more effective pest management in agriculture (Brodmann et al., 2002).

However, the growing use of transgenic crops and biopesticides has raised concerns about the release of DNA through either pollen or degradation of plant biomass to recipient environments. Many tons of free DNA have been shown to be released from pollen, leaves, and fruits in the environment (Dale et al., 2002). The release of DNA in the environment increases the probability of horizontal gene transfers (HGT) in microorganisms by transduction, transformation, and conjugation. These characteristics allow them to rapidly adapt to their environment by acquiring and selecting new genes for survival. Transgenic plant DNA containing a prokaryotic sequence associated with a functional bacterial promoter (CaMV 35S promoter) could cross the genetic barrier that usually prevents plant to microorganism transfer of DNA (Bertolla and Simonet, 1999). The possibility of HGT from transgenic plants has raised many questions with regard to the effects of novel DNA combinations introduced to the environment (Giovannetti, 2003; Dale et al., 2002; Jiang and Paul, 1998). On one hand, HGT might occur in higher organisms such as eukaryotes, although seemingly improbable (Nielsen et al., 1998). On the other hand, a study revealed that the plasmid from Bt expressed Cry1Ab protein in mice and rabbits when injected intramuscularly (Pang, 1994).

The release of exogenous DNA by crops constitutes an important source to the aquatic environment. Thus, an investigation of the occurrence, fate, and persistence of the *cry1Ab* gene in rivers near transgenic corn planted in fields appeared to be relevant for assessing the potential environmental impacts of these bioproducts. The goals of this study were to develop a methodology for extracting DNA from various environmental compartments (soil, sediment, surface water, and biota) and to determine the concentration of the *cry1Ab* gene levels in both transgenic corn and Btk in adjacent aquatic environments. The persistence of this exogenous gene in sediment and surface water in the laboratory was also evaluated.

2. Methods

2.1. DNA extraction in environmental samples

DNA was extracted from sediment, soil, and surface water according to the following approaches. For solid samples (sediment and soil), 100 μL of sterile 100 mM $AlNH_4(SO_4)_2$ was mixed with 500 mg of each sample to limit the coextraction of organic compounds that might block the polymerase chain reactions (PCR) (Braid et al., 2003). An extraction solution containing the detergent, sodium dodecylsulfate (SDS), was added according to specifications of the commercial UltraClean Soil DNA Isolation kit (Mo Bio Laboratories, Solana Beach, CA, USA), which was based on DNA recovery by silica-affinity chromatography. For surface water samples, the pH was adjusted with 200 mM Tris–HCl to pH 8, and 1 g of DEAE-cellulose was added and stirred for 30 min. The DEAE-cellulose was recovered on membrane filter column (30 μm porosity) and washed with 10 mL of 0.1 M NaCl buffered with 25 mM Tris–HCl, pH 8. The DNA was eluted with 2 M NaCl buffered with 25 mM Tris–HCl, pH 8, precipitated with two volumes of ice-cold ethanol for 30 min at −80 °C, and centrifuged at 10,000*g* for 5 min, and the pellet was washed with 70% ice-cold ethanol. The pellet was dried and resuspended in 10 mM Tris–HCl, pH 8.0, containing 1 mM EDTA, and purity was evaluated by absorbances at 230, 260, and 280 nm. When impurities were present (pure DNA has a ratio $A_{260}/A_{230} = 1.7–2.2$), the DNA sample was passed through the commercial UltraClean Soil kit until satisfactory purity was reached.

Various DNA-extraction strategies for environmental samples, such as surface water, soil, and sediment, were evaluated because of the occurrence of inhibitors of PCR (e.g., dissolved humic acids) that contaminate DNA preparations when extracted from field samples. Two in-house and two commercial total DNA extraction procedures were examined. The first in-house method consisted of extracting the samples with SDS and high salt (1.5 M NaCl) in the presence of the cationic detergent, cetyltrimethylammonium bromide (CTAB) (Zhou et al., 1996). The second in-house method was based on selective extraction of DNA on polyvinylpolypyrrolidone columns (Berthelet et al., 1996). For the commercial extraction kits, the SoilMaster DNA Extraction Kit (Epicentre, Madison, WI, USA) and the UltraClean Soil DNA Isolation kit (Mo Bio Laboratories) were used. These methods were evaluated on the basis of the following criteria: time required to obtain DNA, total yield of DNA, and purity of DNA (evaluated by the A_{260}/A_{230} and A_{260}/A_{280} ratios). DNA was quantified by absorbance at 260 nm (Beckman DU70 spectrophotometer). Salmon sperm DNA was used for calibration.

2.2. Detection of cry1Ab gene by fluorescent real-time PCR

A quantitative fluorescent real-time polymerase chain reaction (qPCR) procedure was conducted using the iCycler iQ real-time detection system (Bio-Rad Laboratories, Hercules, CA, USA) with SYBR Green I as the fluorescent dye. The reaction took place in a volume of 50 μL, containing 25 μL of iQ SYBR Green Supermix (Bio-Rad Laboratories), 0.5 μM forward and reverse primers, and 5 μL of test sample. For bacterial *cry1Ab*, universal primers for Bt cry1 were used as previously described (Ben-Dov et al., 1997). For Bt corn (MON810), primers recommended by Kuribara et al. (2002) were used: HSP70 forward primer (5′-GAT GCC TTC TCC CTA GTG TTG A-3′) and *cry1Ab* reverse primer (5′-GGA TGC ACT CGT TGA TGT TTG-3′). DNA from commercial Bt biopesticide (Dipel) and Bt corn grains were extracted using the methods described in Sambrook et al. (1989) and Aljanabi and Martinez (1997) respectively, and used as positive controls. To detect false negatives, DNA from either Bt corn or Bt was added to the extracted DNA samples. The qPCR conditions were 3-min initial denaturation at 95 °C, 40 cycles consisting of a 0.5-min denaturation step at 95 °C, a 1-min annealing step at 59 °C, and a 1-min extension step at 74 °C. Fluorescence at 94 °C at 490 nm excitation and 530 nm emission wavelengths was measured at 80 °C for 10 s. A melt-curve analysis was performed immediately after using 70 cycles of 0.5 °C increments for 10 s, starting at 59 °C and ending at 94 °C. Gel electrophoresis was performed at a 2% agarose concentration at 100 V for 45 min. Staining was done using the fluorescent probe, SYBR Green, at 1X (as recommended by the manufacturer), for 40 min (Molecular Probes, Eugene, OR, USA). Pictures were taken using an UV-transilluminator equipped with a visible filter screen (Advanced American Biotechnology & Imaging, CA, USA).

2.3. Persistence of the cry1ab gene in sediment and surface water

The persistence of the *cry1Ab* gene from Bt corn and Btk in both clay- and sand-rich sediments was studied. DNA extracted from Bt corn was added to 500 mg of sediment to a final concentration of 40 μg/g sediment. Negative controls were spiked with an equivalent amount of sterile distilled water. The spiked samples were thoroughly mixed and incubated in the dark for 1 h at 15 °C to permit adsorption of the DNA to the particles (Franchi et al., 1999). The T_0 samples corresponded to DNA extracted immediately after this adsorption period. The spiked samples were further incubated for 2, 7, 14, 21, 32, and 40 days at 15 °C (15-h light:9-h dark cycle) with humidity adjustment which consisted of

maintaining the total wet volume of the sediment sample throughout the experiment by adding sterile distilled water. The efficiency of DNA extraction at each time of collection was assayed in a duplicate tube containing added DNA sample after 1 h incubation time.

The persistence of the *cry1Ab* gene from both Bt corn and Bt was examined in surface water. Surface water (0.5 L) was split into two 0.25-L samples: one sample was filter-sterilized through a 0.22-μm pore filter, and both samples were spiked with 40 ng/mL of DNA from either Bt corn or Bt and stirred for 1 h at 15 °C in the dark. Negative controls were spiked with distilled water only. The initial (T_0) samples were extracted after the 1-h incubation step, whereas the other samples were incubated for 2, 7, 14, and 21 days at 15 °C (15-h light:9-h dark cycle).

2.4. Field studies near a Bt cornfield

Soil (0.5–1 kg), sediment (0.5–1 kg), and surface water (4 L) samples were collected with a hand shovel (solid samples) or by hand (surface water) adjacent to Bt cornfields before planting (May), 2 weeks after pollination (late August), and 2 weeks after harvest and mechanical mixing of root in the soils (late November/ early December). The cornfield was equipped with a drain pipe that released water into a small creek. The creek connected with a small river (river 1) 2.5 km downstream and this river connected into a larger river (river 2) which is a tributary to the St. Lawrence River (Québec, Canada). The first 20 cm of soil was sampled with a shovel while sediments were collected with a dredge and placed in polyethylene bags. Surface water was collected in 4-L polyethylene bottles. Samples were stored at 4 °C if their processing and analysis occurred within 1 week or at −20 °C (1–10 weeks) for later treatment.

The total amount of DNA extracted from field samples was determined by the Quant-It Picogreen dsDNA Kit (Molecular Probes). Fluorescence was measured at 485 nm (excitation) and 510 nm (emission) using a black 96-well microplate (Titertek, Fluoroskan II). Lambda DNA standard was used for calibration. Data were expressed as μg DNA/g of wet sediment or L of surface water. The *cry1Ab* gene from Bt and transgenic corn (MON810) was determined in soil, sediment, and surface water by qPCR, as described above. The amplification of a single, specific gene product was examined by both melt-curve analysis and agarose (2%) gel electrophoresis for the presence of a single 276-bp (Bt) or 113-bp (Bt corn) band.

2.5. Data analysis

DNA samples were extracted in triplicate ($n = 3$). When the estimated starting amount of gene level was below 100 copies, the replicate values of the PCR assay were summed instead of calculating means, because of the loss of homogeneity of volume (analyte) handling (Bustin and Nolan, 2004). Means were then calculated for replicate field samples ($N = 3$). Trend analysis was conducted using the rank-based Spearman correlation procedure for the persistence experiments and field samples. Significance was set at the $P < 0.05$ level.

3. Results

DNA extraction from complex and dirty environmental samples was typically plagued by poor DNA recovery due to degradation (nucleases) and irreversible nonspecific binding to particles or fouled by organic compounds (e.g., humic or fulvic acids) and metals. Considering these difficulties, various extraction schemes were examined to identify a protocol that could yield a sufficient amount of DNA free of PCR-inhibiting substances in the least amount of time (Table 1). Among the four protocols examined, the UltraClean Soil DNA Isolation Kit was the most satisfactory, as it extracted DNA relatively devoid of contaminants, as determined by the A_{260}/A_{230} ratio, in less than 2 h. The cationic detergent (CTAB) approach using proteinase K digestion gave the best recovery, but the DNA was less pure (fouled by organic contaminants), as indicated by a low A_{260}/A_{230} ratio (<1.7). The recovery of DNA and its purity with other approaches gave intermediary performances between DNA recovery and purity. Recovery of DNA in spiked samples ranged from 10% to 35% in sand (silica)-rich soils and from 0.1% to 15% in clay-rich soils. Recovery of surface water was generally better than that of sediment, being in the 30–60% range. However, dissolved humic acids in surface water were potent inhibitors of the qPCR. Thus, DNA extracted from surface water often required further clean-up steps using the UltraClean Soil DNA Isolation Kit.

DNA was found in detectable quantities in rivers close to cornfields (Table 2). Typically, the levels of extracted total DNA were 0.02–8.5 μg/g for sediment samples and

Table 1
Comparison of DNA yield and purity obtained with different soil/sediment extraction and purification protocols

Extraction procedure	Purification procedure	Time required (h)	DNA yield[a] in agricultural soil (μg/g soil)	DNA yield[a] in freshwater sediment (μg/g sed.)	$A_{260}/_{230}$ ratios in agricultural soil	$A_{260}/_{230}$ ratios in freshwater sediment
Enzymatic (proteinase K)/ detergent) Zhou et al. (1996)	CTAB[b]	6	8.37±0.69	7.91±0.87	1.5	1.6
Enzymatic (lysozymes, proteinase K)/detergent) Berthelet et al. (1996)	PVPP[c] spin columns	8–10	3.06±0.84	2.40±0.26	2.5	1.7
Enzymatic (proteinase k)/ detergent) (Epicentre)	Soil master spin column	3–4	1.00±0.88	4.1±1.9	1.6	1.3
Mechanical/chemical lysis (UltraClean)	Humic acid precipitation, silica membrane spin filter	1.5	2.8 ±4	3.98±0.11	2.2	2.2

[a]DNA yields: mean±SD, $n = 4$.
[b]CTAB, cetyltrimethylammonium bromide.
[c]PVPP, polyvinylpolypyrrolidone.

Table 2
Amounts (μg/g or L) of total extracted DNA

Site	Concentration of extracted total DNA (μg/g sediment or soil)		
	May 2004	August 2004	November 2004
(a) *In soil/sediment samples*			
Soil (wild corn)	1.6±0.21[a]	3±0.63	1.8±0.41
Soil (Bt corn)	1.52±0.22	8±1.2	3.1±0.33
2 m upstream	0.03±0.0054	—	—
2–10 m downstream	3±0.23	3±0.42	1.5±0.12
2.5 km downstream (at confluence with connecting river 1)	1.5±0.32	0.02±0.0053	1.3±0.12
8.5 km downstream (in connecting river 1)	1±0.21	1±0.14	3±0.53
16.5 km downstream (at confluence with connecting river 2)	1±0.13	0.5±0.052	0.6±0.074
82 km downstream (at confluence with St. Lawrence River)	1.6±0.22	1.3±0.23	0.07±0.12
Site	Concentration of extracted total DNA (μg/L surface water)		
	May 2004	August 2004	November 2004
(b) *In surface water samples*			
2 m upstream	ND[b]	NA[c]	NA
Drain	0.013±0.0012[a]	0.011±0.0021	ND
2–10 m downstream	0.001±5.3^{e-4}	0.014±0.0012	0.01±0.0013
2.5 km downstream (at confluence with connecting river 1)	ND	0.023±0.0041	0.015±0.0021
8.5 km downstream (in connecting river 1)	ND	0.012±0.0012	0.011±0.0013
16.5 km downstream (at confluence with connecting river 2)	0.003±5.3^{e-4}	0.023±0.0032	0.022±0.0042
29.5 km downstream (in a connecting river 2)	ND	0.008±0.0013	0.015±0.0041
82 km downstream (at confluence with St. Lawrence River)	ND	0.03±0.0062	0.001±0.22^{e-4}

[a]Mean±standard deviation.
[b]ND, not detectable.
[c]NA, not analyzed.

1–30 ng/L for surface waters. In sediment samples, the amount of DNA extracted did not vary significantly with sampling time ($P>0.1$). In surface water samples, DNA levels were significantly higher ($P<0.05$) in August (after pollen release) and in November (just after mechanical mixing of the corn stems and roots; i.e., corn rhizospheres and stems were mixed mechanically before the winter season), compared with before planting in May. The total amount of DNA in surface water and sediment samples tended to be lower at the farthest sites, near the confluence of the Richelieu and St. Lawrence rivers, rather than at those close to the cornfields.

Transgenic *cry1Ab* was readily detected in Bt corn with the selected primers using qPCR (Fig. 1). Using universal primers for the *cry1* genes for Bt also proved successful (results not shown). A highly significant negative relationship ($R\geqslant0.99$; $P<0.001$) was obtained between the threshold cycle (the PCR cycle when fluorescence was detected above background indicative of significant DNA synthesis) and the initial amount of genomic DNA. PCR efficiency was typically good, ranging from 80% to 95%. Efficiency values above 110% represent nonspecific amplifications and primer dimers, while efficiency values lower than 90% represent poor primer design with the cycling temperature used (Brisson et al., 2004). The slope of the threshold cycle was linear down to 670 pg of genomic DNA, with a detection limit of about 200 pg of genomic DNA (Fig. 1A). A melt-curve analysis of the PCR products revealed major peaks at 83 °C for Bt corn (Fig. 1B) and at 84.5 °C for Bt. Agarose gel electrophoresis revealed single bands at 113 bp for Bt corn and at 276 bp for Bt (Fig. 2).

The sediments and surface waters were spiked with genomic DNA from either Bt corn or Bt to evaluate their respective degradation rates in these aquatic environments (Figs. 3 and 4). In sediment samples, the water fraction was adjusted to the initial volume every 2 days to maintain the same proportion of water to sediment for the duration of the experiment. Recovery of DNA was constant albeit fairly low, ranging between 30% and 35% at T_0 and for all extraction times (40 days). The content of the *cry1Ab* gene from Bt corn decreased quickly within the first few days, but it was still detected 21 days later. The half-life ($t_{1/2}$) of the *cry1Ab* gene from Bt and Bt corn was estimated at 1.7 days for both types of sediment (i.e., clay- and sand-rich sediments). A Spearman rank correlation analysis between levels of the *cry1Ab* gene and incubation time was negatively correlated, with $R=-0.82$; $P<0.01$ for both clay and sand types. The degradation rate of the *cry1Ab* gene from Bt did not differ significantly from the *cry1Ab* from Bt corn (results not shown). About 1% of the initial amount of DNA remained in the sediment samples after 40 days. The gene *cry1Ab* in surface water also persisted for many weeks (Fig. 4). More than half the initial amount of DNA was degraded after 7 days but it was still detected 21 days later. The estimated $t_{1/2}$ was 14.3 days for both *cry1Ab* gene fragments. The removal of microorganisms by

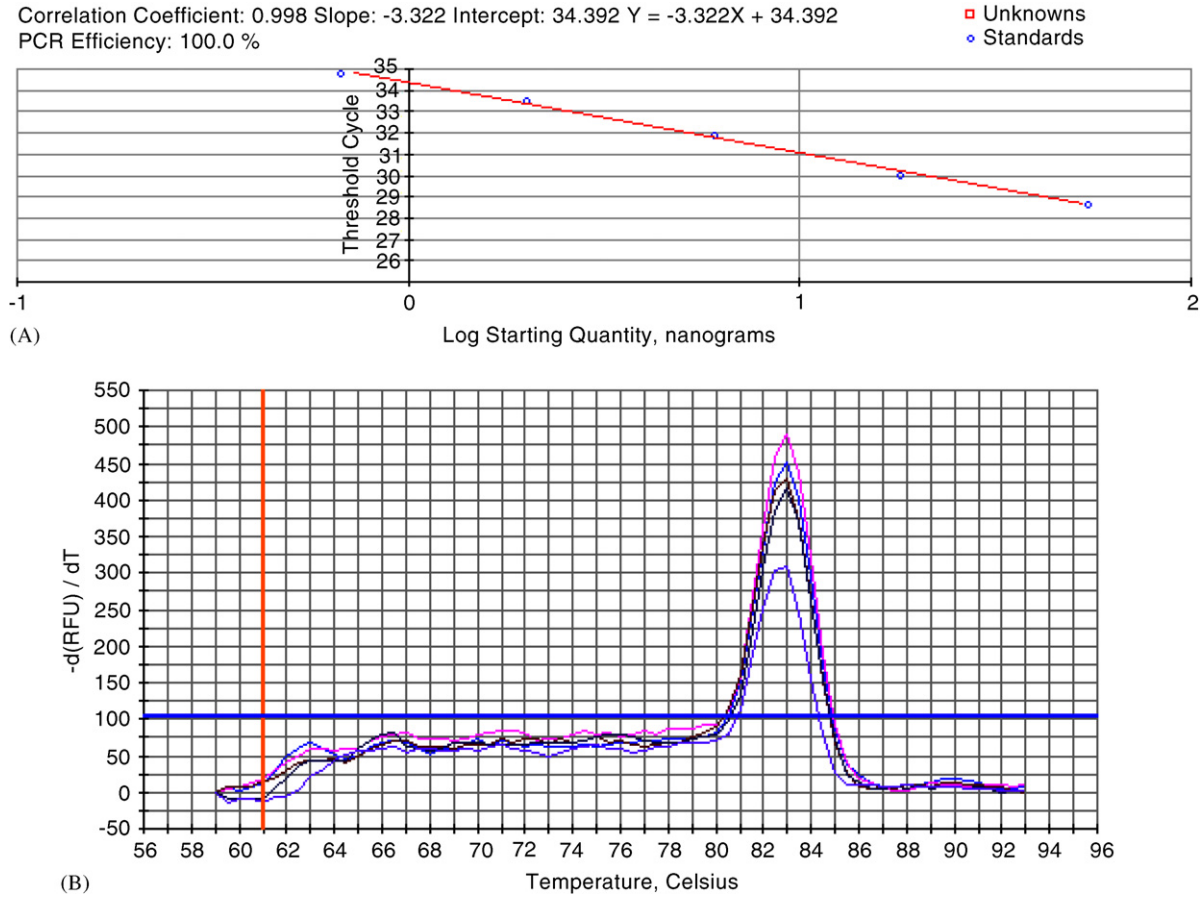
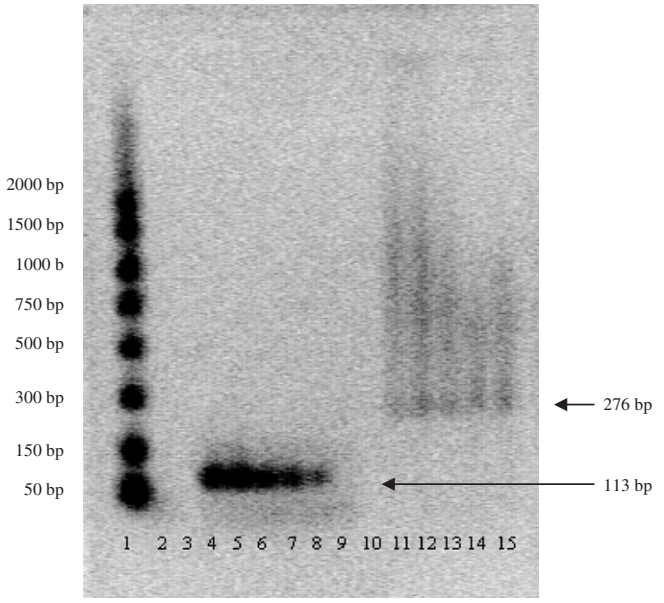


Fig. 1. *cry1Ab* gene from Bt corn using quantitative real-time PCR. Threshold cycle determination (A) and melt-curve analysis (B) with increasing amounts of Bt corn DNA. For melt-curve analysis, the ordinate scale is defined as change in relative fluorescence units or d(RFU) per change of temperature (dT).



1: Standard	4: 54 ng Bt corn DNA	7: 2 ng Bt corn DNA	10: empty	13: 6 ng Bt DNA
2: Blank-Bt corn	5: 18 ng Bt corn DNA	8: 0.67 ng Bt corn DNA	11: 54 ng Bt DNA	14: 2 ng Bt DNA
3: empty	6: 6 ng Bt corn DNA	9: Blank Bt DNA	12:18 ng Bt DNA	15:0.67 ng Bt DNA

Fig. 2. Agarose gel electrophoresis of Bt corn and Bt *cry1Ab* amplicons of positive controls.

filtration through a sterile 0.2-μm-pore filter increased the persistence of the *cry1Ab* gene at the end of the incubation period when its concentration was about two to five times more abundant than that in raw (unfiltered) water at the end of the experimental period (day 40). However, the half-life of the *cry1Ab* gene was not affected by filtering surface water samples.

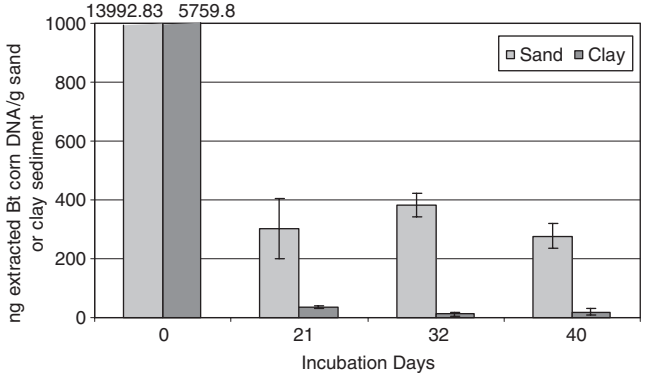


Fig. 3. Persistence of *cry1Ab* gene from Bt corn in clay- and sand-rich sediments with moisture content adjustment. The data represent the mean ± standard error from $n = 3$ replicates.

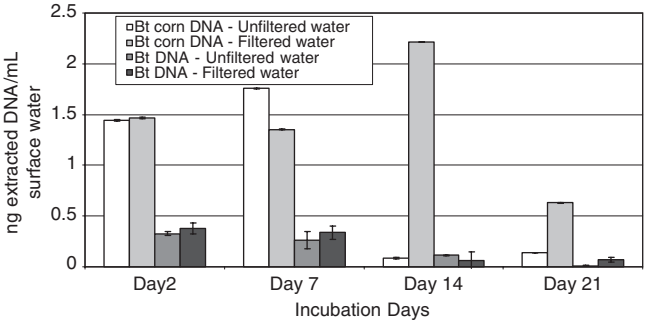


Fig. 4. Persistence of *cry1Ab* gene in surface waters. Surface waters were spiked with DNA from Bt corn or Bt and allowed to incubate at 15 °C for several days. The data represent the mean and standard error from $n = 3$ replicates.

Soil, sediment, and surface water samples were collected in May, August, and and December of 2003 and in May, August, and November of 2004 (Tables 3 and 4). The sampling months were chosen in relation to the normal activities related to corn cultivation and harvest. Samples collected in May reflect the baseline levels before seeding corn while those collected in August occurred after pollen release (2 weeks). The post-harvest and stem/root mixing samples were collected in late November/early December. The *cry1Ab* transgene was detected at higher levels in soil near the roots of the Bt corn, although *cry1Ab* was also found near the roots of the non-Bt (native) corn because the two crops were grown in the same area (i.e., they were grown in the same soil because of mixing after the harvest period). The *cry1Ab* transgene was sometimes found at the confluence of the Richelieu and St. Lawrence rivers, indicating contamination by agricultural transgenic DNA. During the May sampling period there was no direct source of *cry1Ab* other than the soil containing roots and stems from the previous year. However, there was an increase in *cry1Ab* in May 2003 and 2004, especially 2.5 km downstream of the fields. Indeed, over the 2-year sampling period, the highest levels of the transgene were found in May and August, and not at the post-harvest sampling time, in both sediment and surface water. Moreover, in 2003, *cry1Ab* levels were significantly higher in May than in August (Mann–Whitney U test, $P < 0.05$) in sediments, while no significant difference was found in surface water ($P > 0.1$). In 2004, samples from both the sediment and the water compartments were not significantly different between the May and the August sampling times. Sediments contained significantly more *cry1Ab* DNA than did surface water (Mann–Whitney U test, $P < 0.01$), indicating that sediment was the primary sink for these genes in this environment. Indeed, *cry1Ab* DNA was about 100-fold more abundant in the sediment compartment than in the surface water. Nevertheless, levels of *cry1Ab* DNA in sediment and surface water were significantly correlated ($R = 0.83, P = 0.04$).

Table 3
Detection of *cry1Ab* transgene from Bt corn in sediment and soil near a Bt cornfield

Site	Concentration of extracted Bt corn DNA (ng/g sediment or soil)					
	May 2003	August 2003	December 2003	May 2004	August 2004	November 2004
Soil (wild corn)	1.9 ± 0.2[a]	2.4 ± 0.1	ND[b]	ND	2.6 ± 0.2	ND
Soil (Bt corn)	6.9 ± 1	0.4 ± 0.2	ND	ND	84 ± 14	ND
2 m upstream	7.6 ± 1	0.6 ± 0.2	1.5 ± 0.25	ND	NA	NA[c]
2–10 m downstream	8.4 ± 1.5	2.2 ± 0.5	0.1 ± 0.2	ND	ND	2.6 ± 0.5
2.5 km downstream (at confluence with connecting river 1)	120 ± 22	1.4 ± 0.4	ND	1.3 ± 0.3	ND	2.1 ± 0.21
8.5 km downstream (in river 1)	ND	ND	ND	ND	ND	ND
16.5 km downstream (at confluence with connecting river 2)	ND	ND	ND	1.3 ± 0.2	ND	2.6 ± 0.2
82 km downstream (at confluence with St. Lawrence River)	1.0 ± 0.2	0.6 ± 0.2	ND	ND	0.8 ± 0.2	0.7 ± 0.2

[a]Mean ± standard deviation.
[b]ND, not detectable.
[c]NA, not analyzed.

Table 4
Detection of *cry1Ab* transgene from Bt corn in surface water near a Bt cornfield

Site	Concentration of extracted Bt corn DNA (ng/mL surface water)					
	May 2003	August 2003	December 2003	May 2004	August 2004	November 2004
2 m upstream	0.70 ± 0.11[a]	$0.005 \pm 5 \times 10^{-4}$	ND[b]	ND	NA[c]	NA
Drain	0.06 ± 0.005	0.16 ± 0.02	$0.002 \pm 1 \times 10^{-4}$	ND	ND	ND
2–10 m downstream	0.02 ± 0.003	0.02 ± 0.002	ND	$0.006 \pm 1 \times 10^{-4}$	ND	ND
2.5 km downstream (at confluence of connecting river 1)	0.01 ± 0.002	0.47 ± 0.05	ND	ND	$0.005 \pm 5 \times 10^{-4}$	ND
8.5 km downstream (in connecting river 1)	ND	ND	ND	ND	0.018	ND
16.5 km downstream (at confluence of connecting river 2)	ND	$0.004 \pm 3 \times 10^{-4}$	ND	0.001	$0.003 \pm 2 \times 10^{-4}$	$0.001 \pm 1 \times 10^{-4}$
29.5 km downstream (in a connecting river 2)	0.010 ± 0.001	0.011 ± 0.002	ND	ND	$0.007 \pm 5 \times 10^{-4}$	ND
82 km downstream (at confluence of the St.Lawrence river)	ND	$0.005 \pm 3 \times 10^{-4}$	ND	ND	ND	$0.009 \pm 5 \times 10^{-4}$

[a]Mean ± standard deviation.
[b]ND, not detectable.
[c]NA, not analyzed.

4. Discussion

Analysis and quantitation of genes were usually performed by melt-curve and by threshold-cycle analyses with real-time PCR. Melt-curve analysis provided additional information on the presence of primer dimers and other amplicons. Linear relationships between the threshold cycle or the area under the melt curve and the addition of Bt and Bt corn DNA were observed. Based on the estimated copy number of genomic DNA in corn, the calculated detection limit of the qPCR methodology was 40 genomic copies. The theoretical limit for the quantitative determination of genes was 35 genomic copies, with a detection limit between 1 and 10 genomic copies for an optimized PCR method (Hübner et al., 2001). The present methodology was, therefore, able to attain the theoretical limit, as 0.2 ng of Bt corn DNA (about 40 corn genomic copies) gave a positive signal.

The practice of growing transgenic crops and the use of biopesticides have prompted the need to monitor the fate of genetically modified genes introduced in the environment, especially in aquatic ecosystems where high dispersal potential exists. The continued presence of transgenes in the environment augments the possibility of gene transfer events to biota with potentially adverse effects to the genetic biodiversity. The extent to which DNA has been shown to adsorb to particles and colloids (i.e., humic and fulvic substances) greatly affects its persistence in the environment, its availability to nuclease attack by microorganisms, and its horizontal transfer (Stotzky, 2000; Romanowski et al., 1991). The binding of DNA with clay particles such as montmorillonite and kaolinite significantly increased its persistence in various environments (Franchi et al., 1999). For example, clay moisture content and temperature are factors that influenced the half-life ($t_{1/2}$) of particular genes (Degand et al., 2002: Romanowski et al., 1991; Lorenz and Wackernagel, 1987). In the present study, the *cry1Ab* gene from Bt corn persisted for 40 days in sediment, which corroborates the findings of Widmer et al. (1996), who detected transgenic DNA in microcosms 130 days after its introduction. In another study, transgenic DNA and native DNA from sugar beet were detected in soils after 25 days in the field (Degand et al., 2002). Transgenic *cry1Ab* derived from Bt cornfields was found in nearby streams and rivers and sometimes several kilometers downstream. The persistence of these transgenes in the receiving environment could increase the likelihood of in situ HGT. In vitro experiments on HGT have shown that transgenic plant DNA was able to transform *Acinotobacter calcoaceticus* and restore an intact gene in the recipient cell, which was then able to express resistance to the antibiotic kanamycin (Gebhard and Smalla, 1998). Moreover, clay-bound DNA retains the ability to transform competent cells for many weeks (Stotzky, 2000; Khanna and Stotzky, 1992). Intramuscular injections of Bt plasmid into mice and rabbits successfully produced Cry1Ab endotoxin as evidenced by the presence of Cry1Ab protein immunoglobulins (Pang, 1994), indicating that prokaryotic DNA could be actively expressed in mammalian cells. Injections of plasmid DNA encoding either the G protein or N protein from hemorrhagic septicaemia virus was also successful in immunizing rainbow trout against either the G or N proteins (Lorenzen et al., 2005).

The increased presence of the *cry1Ab* transgene but not the *cry1Ab* gene from Bt in May before sowing of Bt corn seed was of concern, as it suggests that the transgene survives over the winter months. Whether the transgene was associated with decaying corn roots, sediment particles,

bacteria, or other microorganisms remains to be established. DNA was produced in extracellular environments by bacteria in aquatic microcosms. Genetically altered strains of *Escherichia coli*, *Pseudomonas aeruginosa*, *Pseudomonas cepacia*, and *Bradyrhizobium japonicum* were able to produce extracellular nucleic acids where the production and release of extracellular (i.e., cell-free) DNA were more strongly influenced by physicochemical factors than by biotic factors (Paul and David, 1989). Furthermore, rates of extracellular DNA production were greater in fresh than in marine waters, and ambient microbial populations were able to utilize readily materials released by these organisms. However, the levels of Cry1Ab protein in our samples were below the detection limit most of the time, although it was detected at concentrations ranging from 0.1 to 1 ng/g or ng/mL in sediment and surface water, respectively (Douville et al., 2005). This finding suggests that environmental concentrations were low but the null hypothesis (i.e., that the *cry1Ab* transgene was not expressed in aquatic environments) cannot be accepted at the present time and further research and monitoring efforts are required. It would also be of interest to sequence the amplified fragments to check for changes in the nucleotide sequences in the field samples. On the assumption that the biotechnology industry is expanding, the development of monitoring strategies to characterize environmental exposure and to examine the effects of products such as the *cry1Ab* gene from transgenic corn or Bt biopesticide applications are fully justified. Increasing use of transgenes has the potential to release biologically active materials into the environment to the extent that nontarget organisms might suffer adverse effects from exposure. Indeed, continuous introduction of technology-driven exogenous genes in various compartments of the biosphere may influence genetic biodiversity and this issue merits sustained attention.

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